The effects of various membrane physical–chemical properties on the aggregation kinetics of insulin

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Abstract

In a simplified approach to the in vivo situation, where pathogenic fibrillar protein deposits are often found associated with cellular membranes, the aggregation kinetics of insulin in the presence of various model biomembranes were investigated using the Thioflavin T (ThT) fluorescence assay. The lipid dynamics near the gel–fluid transition, the chain length of saturated lipids and the presence of DOPE or DOPS in DOPC-vesicles modulate the aggregation kinetics of insulin in an indifferent, an aggregation-accelerating or an aggregation-inhibiting manner, subtly depending on the pH-value and the presence of salt. The rate of insulin aggregation in bulk solution dominates the overall aggregation process in most cases at low pH, where the lipid additives exert no effect on the aggregation kinetics. The occurrence of dynamic line defects near the gel–fluid transition temperature of DSPC facilitates a partial membrane insertion of the protein, which in turn shields exposed hydrophobic protein patches from intermolecular association and hence inhibit aggregation. An exclusively aggregation-accelerating effect was observed in the presence of 0.1 M NaCl for all lipid additives investigated, which is likely due to an enhanced surface accumulation of the protein. Apart from weak dipole–dipole, dipole–monopole and hydrogen bonding interactions, the release of curvature elastic stress in mixed DOPC/DOPE-membranes and preferred interactions of insulin with carboxylic groups in DOPC/DOPS-membranes favour an increased surface accumulation. At neutral pH, a partial insertion of insulin into the lipid bilayer is favoured, which accounts for the aggregation-inhibiting effect of all lipid bilayer systems studied except those containing DOPS. Generally, the extent of inhibition increases with the lipid chain length and the extent of curvature stress in mixed unsaturated lipid membranes and also when the gel–fluid transition temperature of pure phospholipids is approached. The accelerating effect of DOPS on the aggregation of insulin under net electrostatic repulsion at pH 7.4 remains to be elucidated, yet, it might result from increased surface accumulation and/or faster/more extensive unfolding of the protein without a subsequent membrane insertion. These results demonstrate that a delicate interplay between different physical and chemical properties of lipid membranes has to be taken into account in a detailed discussion of membrane-associated protein aggregation phenomena.

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1. Introduction

The physical and chemical properties of biological membranes are essentially modulated by the composition of the constituent lipids, which finally manifests in an environmentally adapted regulation of the func-
tion of membrane proteins (Zidovetzki, 1997; Epand, 1997). Recently, it has become evident that interactions with biological membranes also play a crucial role in the aggregation of amyloidogenic proteins in vivo. On the one hand, it has been shown that aggregated conformations of amyloidogenic proteins may be cytotoxic to cultured cells (Bucciantini et al., 2002; Bucciantini et al., 2004; Kayed et al., 2003). Additionally, in vitro studies have demonstrated that amyloid aggregates can disintegrate the structure of model biomembranes by, e.g., enhancing their permeability and thus altering the natural balance of ion fluxes in a pathogenic manner (Stefani and Dobson, 2003). On the other hand, membrane induced conformational changes have been documented for some amyloidogenic proteins, which correlates with an accelerated protein aggregation and fibrillation (Zhu et al., 2003; Zhao et al., 2004; Knight and Miranker, 2004) imply that the underlying modes of interactions are critically modulated by the membrane lipid composition.

Among the proteins which are prone to aggregation and fibrillation, insulin has become a model system due to its medical importance and the wealth of available data. Insulin, which forms hexamers under physiological conditions, aggregates under conditions like low pH, high temperatures or stirring in the presence of hydrophobic surfaces (Chiti and Dobson, 2006; Smirnovas et al., 2006; Whittingham et al., 2002; Sluzky et al., 1991; Ahmad et al., 2004; Podesta et al., 2006; Dzwolak et al., 2004, 2005; Jansen et al., 2005; Grudzielanek et al., 2005, 2006). At pH 2, insulin forms dimers in water, and the addition of particular cosolvents, such as 20 wt% ethanol, does not only render insulin monomeric at pH 2, but also affects the aggregation kinetics (Dzwolak et al., 2005). The aggregation and fibril formation of this protein in bulk solution has been investigated with various methods like FTIR and fluorescence spectroscopy as well as atomic force microscopy (AFM) (Dzwolak et al., 2005; Jansen et al., 2005) but detailed studies yielding information on the effect of the presence of various types of lipid interfaces are still largely lacking.

In the recent literature, several studies on protein–membrane systems indicate that the aggregation of amyloidogenic proteins in the presence of lipid membranes proceeds via a stepwise aggregation mechanism (Gorbenko and Kinnunen, 2006). These steps usually comprise the adsorption of the protein at the membrane surface, a conformational change of the protein (often via partial unfolding) and subsequently nucleation and fibril growth. The relevance of particular modes of interaction as well as the underlying mechanisms can be systematically evaluated by using properly chosen model membranes with a well-defined composition, which is the case for, e.g., unilamellar lipid vesicles. In the present study, the following physical–chemical aspects have been investigated more in detail.

(i) Lipid dynamics: The gel–fluid phase transition – the most prominent among the thermotropic transitions of lamellar hydrated lipid bilayers – is accompanied by a significant increase of the dynamics of the lipid molecules. Lipid domains in the gel and in the fluid state coexist at the transition temperature $T_m$, where the appearance of line defects is maximal due to the dynamic interconversion between the two kinds of domains. It is well known that the maximum occurrence of line defects at $T_m$ correlates with a maximum permeability of membranes towards many substances (Lasic, 1993).

In an analogous fashion, it is reasonable to investigate the aggregation behaviour of amyloidogenic proteins near $T_m$ to gain information about the relevance of line defects as a driving force for a potential (partial) membrane insertion of the protein. (ii) Lipid chain length: A variable acyl chain length of phospholipids with the same head group does not only influence $T_m$ (generally, $T_m$ increases with increasing chain length), but also determines the thickness of the hydrophobic interior of lipid bilayer. It has been observed that the insertion of proteins into membranes can, among others, be modulated by the extent of “hydrophobic matching” (Jensen and Mouritsen, 2004). “Hydrophobic mismatch” describes the situation when the thickness of the hydrophobic portion of an isolated bilayer differs from that of a membrane protein. If the mismatch is not too high, “matching” can be accomplished by the compression or stretching of the acyl chains in order to avoid an energetically unfavourable “mismatched” situation. Experimentally, one can predetermine the extent of “matching” by choosing lipids of different acyl chain length (in this study: C$_{14}$–C$_{18}$) and consequently investigate the interaction of the protein with the respective membrane (e.g., Harroun et al., 1999). This approach is especially motivated here by the fact that the formation of transmembrane pores after insertion has been proposed as a mechanism for the membrane disruption by amyloidogenic proteins (Caughey and Lansbury, 2003). (iii) Curvature elastic stress: Phospholipids with a small ratio of head group/acyl chain cross section (e.g., phosphatidylethanolamines) tend to form inverse-hexagonal phases in aqueous solution. The presence of
such lipids in planar lipid bilayers results in a frustrated bilayer that experiences curvature elastic stress. The insertion of proteins into curvature-stressed membranes can be thermodynamically favourable, because it causes the membrane to locally bend and release curvature elastic stress (Stubbs and Slater, 1996; Hui, 1997). The investigation of the effect of curvature stress with respect to the aggregation behaviour of amyloidogenic proteins is motivated by the observation that the release of curvature stress has been reported to be a major driving force for the interaction of α-synuclein with highly curved unilamellar vesicles (Nuscher et al., 2004). (iv) Charge effect: The insertion of charged lipids (e.g., negatively charged phosphatidylserines at physiological pH) modulates the electrostatic potential of lipid membranes. According to the diffuse layer theory, the concentration of counterions in solution is maximal directly at an electrically charged surface and approaches the value in the bulk with increasing distance. The surface accumulation of amyloidogenic proteins due to attractive electrostatic interactions is being discussed as a major cause for the aggregation-accelerating effect of negatively charged lipid membranes (Gorbenko and Kinnunen, 2006). Besides, the low local pH near the surface of such membranes seems to favour the partial unfolding – and hence formation of aggregation-prone intermediates – of adsorbed protein molecules. The influence of electrostatic interactions on protein aggregation processes in the presence of charged lipid membranes can be evaluated by the effect of charge screening, i.e., by the addition of salts.

The occurrence of fibrillar tissue-associated insulin deposits during the treatment of diabetes has provided first clues as to the critical involvement of membrane constituents in the development of local, iatrogenic insulin-dependent amyloidosis (Swift et al., 2002; Störkel et al., 1983; Dische et al., 1988). To date, however, only a few and partly contradictory studies on the interaction of insulin with lipid membranes have been published. For native insulin, weak interactions with phosphatidylcholines have been evidenced. Those interactions seem to be mostly electrostatic in nature and may also comprise the formation of hydrogen bonds with the lipid head groups (Omodeo-Sale et al., 1987; Pedersen et al., 1999; Fernández and Berry, 2003). Furthermore, enhanced electrostatic interactions of insulin with negatively charged model membranes have been demonstrated, which are accompanied by vesicle fusion on the one hand (Farias et al., 1986) and supposed to manifest in an accelerated fibrillation of the protein on the other hand (Zhao et al., 2004). Besides, the penetration of lipid monolayers by native insulin has been documented, the extent of which seems to correlate with the degree of unsaturation of the lipids and hence the membrane fluidity (Schwinke et al., 1983). Irrespective of the exact lipid composition, an exclusively aggregation-inhibiting effect of lipid additives – in non-vesicular form, however – has been reported under conditions where an interface-induced unfolding and aggregation of the protein is usually observed (Hansen et al., 1986). Finally, an FTIR-study on the aggregation behaviour on insulin under conditions resembling those in the current study (pH 1.6 at 60°C) indicates an accelerated surface aggregation with respect to the bulk aggregation under both electrostatic attraction and repulsion (Sharp et al., 2002).

Given the rather unsystematic information available about the effects of lipid membranes on the insulin aggregation process, the current study aims at elucidating the relative importance of distinct types of insulin–membrane interactions. To this end, careful tuning of the physical and chemical properties of lipid membranes was accomplished by the choice of model membranes with respect to the relevance of lipid dynamics (DSPC near Tm), hydrophobic bilayer thickness (DMPC, DPPC, DSPC), curvature elastic stress (DOPC/DOPE-mixtures) and charge (DOPC/DOPS-mixtures). The Thioflavin T (ThT) fluorescence assay was used to monitor the fibrillation kinetics of insulin in the presence of the various model membranes in real time, which in turn provides first important clues as to factors that accelerate, inhibit or have no effect on the insulin aggregation process.

2. Materials and methods

2.1. Materials

Bovine insulin was purchased from Sigma (Steinheim, Germany) and used without further purification. Thioflavin T was from Merck (Darmstadt, Germany), and all lipids were from Avanti Polar Lipids (Alabaster, AL). All other reagents in analytical grade were from Sigma. Deionized water was used for preparing aqueous solutions throughout this study.

2.2. Preparation of lipid vesicles

Lipid films of specified compositions were prepared by mixing lipid stock solutions (10 mg/mL) in chloroform and in chloroform/methanol 10:1 (vol/vol) (for DOPS), respectively, drying in a gentle stream of nitrogen and removal of the residual chloroform at 0.1 mbar overnight. The dry lipid films were rehydrated at a nom-
inal concentration of 1.72 mM (for the ThT-assay) or 8.63 mM (for DSC measurements) in HCl, pH 1.9, and in 10 mM phosphate buffer, pH 7.4, respectively (both with and without 0.1 M NaCl). Following five cycles of freeze–thawing, large unilamellar vesicles for the ThT-assay were prepared by 21 cycles of extrusion through polycarbonate membranes with 100 nm nominal pore size (MacDonald et al., 1991) and immediately used for the fluorescence measurements.

2.3. Fluorescence spectroscopy

All fluorescence measurements were performed in photon counting mode on a K2 multifrequency phase and modulation fluorometer (ISS, Urbana, IL), using thermostatted 1 cm square quartz cuvettes (Hellma, Mülheim, Germany).

2.4. ThT-assay of aggregation

Insulin (1%, w/w) and the fibril-specific dye ThT were dissolved in 10 mM HCl and 10 mM phosphoric acid, respectively, at a protein to dye molar ratio of 5:1. Upon 10-fold dilution with deionized water or 10 mM phosphate buffer, the samples were pH-adjusted to 1.9 and 7.4, respectively. Where specified explicitly, the solvents also contained 0.1 M NaCl in order to examine the consequences of charge screening. For the fluorescence measurements, the ThT-containing protein samples were mixed 1:1 (vol/vol) with the unilamellar vesicle preparations in the same respective solvent to finally yield 0.05% (w/w) insulin, 17\%H262 M ThT and 860\%H265 M lipid ([insulin]:[ThT]:[lipid] = 1:0.2:10). Control samples without lipid, but otherwise identically prepared, were used in order to exclusively investigate the bulk aggregation behaviour of the protein. The emission intensity $I$ at 482 nm was recorded as a function of time $t$ upon excitation at 450 nm at 60\°C. Additional measurements at constant temperatures between 52 and 68\°C were performed with DSPC-containing samples after having confirmed by DSC that the main transition temperature of the pure lipid is within this temperature range. All samples were agitated continuously using a magnetic stirring bar.

The kinetic traces were empirically fitted to a sigmoidal equation with the Origin 6.0 software package (Microcal, Southampton, MA):

$$I = I_1 + \frac{I_0 - I_1}{1 + (\exp(t - t_0)/g)},$$

where $I_0$ and $I_1$ are the initial and final fluorescence plateau values, $t_0$ denotes the half-time for aggregation at the point of inflection, and $g$ is a parameter describing the magnitude of the increase of the signal. The $t_0$-values were finally used as empirical parameters for characterizing the rates of aggregation (Knight and Miranker, 2004). The error bars within a particular series of experiments represent statistical errors of at least two independent measurements each, whereas variations among the $t_{0.5}$-values for different series of experiments are due to different insulin batches used (Brange et al., 1997).

2.5. DSC measurements

DSC thermograms of multilamellar DSPC-vesicles at 8.63 mM in 0.1 M NaCl (pH 1.9) and 10 mM phosphate buffer/0.1 M NaCl (pH 7.4) were recorded on a VP DSC microcalorimeter (Microcal, Northampton, MA). The sample and reference cells were filled with 0.54 mL of the lipid sample and degassed. Upon 15 min of equilibration at 5\°C, four DSC scans were successively performed up to 90\°C at a scan rate of 40\°C/h in order to check the reversibility of the lipid phase transition. The DSC thermograms were concentration-normalized, baseline-corrected with the Origin 7.0 software (Microcal, Northampton, MA) and analyzed with respect to the gel–fluid transition temperature $T_m$ and the full width at half height (FWHH) of the DSC peak.

2.6. AFM measurements

Selected insulin samples (controls as well as insulin samples containing a 10-fold molar excess of lipid vesicles) were prepared and incubated at 60\°C with constant stirring like for the ThT-assay, albeit lacking ThT. Following overnight incubation – corresponding to complete aggregation – the samples were diluted with deionized water to a final protein concentration of 0.001% (w/w). Twenty microlitres were applied onto freshly cleaved muscovite mica and allowed to dry. The data were acquired in tapping mode on a MultiMode SPM AFM microscope equipped with a Nanoscope IIIa Controller from Digital Instruments (Mannheim, Germany). As AFM probes, Silicon SPM Sensors “NCHR” (force constant, 42 N/m; length, 125 mm; resonance frequency, 300 kHz) from Nanosensors (Neuchâtel, Switzerland) were used.

3. Results

Fig. 1 presents representative examples of kinetic traces from the ThT-assays for the aggregation of insulin in the presence of lipid vesicles. For the purpose of
Fig. 1. Representative time courses of the ThT emission intensity for the aggregation of 0.05% (w/w) insulin in the presence of lipid vesicles with a 10-fold molar lipid excess in 10 mM Na-phosphate, pH 7.4, with 0.1 M NaCl each. Upper left panel: Dependence on the gel–fluid phase transition of DSPC ($T_m = 54.1 \degree C$). Upper right panel: Dependence on the chain length of various unsaturated phosphatidylcholines at 60 $\degree C$. Lower left panel: Dependence on the fraction of DOPE in mixed DOPC/DOPE-vesicles at 60 $\degree C$. Lower right panel: Dependence on the fraction of DOPS in mixed DOPC/DOPS-vesicles at 60 $\degree C$. Protein samples without lipid, but under otherwise identical conditions, were used as controls.

clarity, the examples shown in Fig. 1 are restricted to the aggregation of insulin in 10 mM Na-phosphate, pH 7.4 with 0.1 M NaCl, only. The temporal evolution of the ThT emission intensity exhibits features which are typically assigned to a nucleated growth aggregation mechanism, i.e., a lag phase that is followed by a sigmoidal increase to a plateau value (Harper and Lansbury, 1997). In the following, the insulin aggregation kinetics will be discussed in terms of the times for half-maximum aggregation, $t_{0.5}$ as an empirical kinetic parameter.

3.1. Aggregation kinetics around $T_m$ of DSPC—probing lipid dynamics

The temperature dependence of the aggregation kinetics of insulin in the presence of DSPC-vesicles was analyzed at pH 1.9 and 7.4 with 0.1 M NaCl within the respective temperature range of the gel–fluid phase transition of DSPC. The DSC thermograms (Fig. 2a) display a single peak each that corresponds to the gel–fluid phase transition of DSPC, yielding $T_m$-values of 66.8 $\degree C$ at pH 1.9 and 54.1 $\degree C$ at pH 7.4, respectively, and FWHH-values of 7.1 $\degree C$ at pH 1.9 and 0.5 $\degree C$ at pH 7.4, respectively. The increase of $T_m$ with decreasing pH-value is well documented in the literature and is ascribed, among others, to the stabilization of the membrane in the headgroup region due to the formation of transient intermolecular hydrogen bonds between neighbouring protonated phosphate groups (Koynova and Caffrey, 1998). Apart from the higher $T_m$ at acidic pH, the DSC thermogram for DSPC at pH 1.9 is markedly broadened, ranging from $\sim$58 to $\sim$69 $\degree C$, and asymmetric.

Expectedly, the time for half-maximum aggregation $t_{0.5}$ decreases with increasing temperature at both pH-values in the absence of lipid vesicles, i.e., the aggregation of the protein accelerates with increasing temperatures (Fig. 2b). Furthermore, the aggregation process is slightly slower at neutral pH compared to acidic pH, which is likely to be attributed to a stabilization of the protein due to hexamer formation at pH 7.4, the dissociation of which has been proposed to be rate limiting for the aggregation process under these conditions (Ahmad et al., 2003).

In contrast, the temperature dependence of $t_{0.5}$ in the presence of DSPC-vesicles exhibits a significant maximum at $\sim$62 $\degree C$ at pH 1.9 and $\sim$54 $\degree C$ at pH 7.4, respectively, i.e., the maximum inhibition of the protein aggregation is observed near or at $T_m$. Compared to the respective control measurement (without lipid), a significant inhibition of the protein aggregation is additionally observed over the entire temperature range at pH 7.4, whereas the inhibitory effect of the lipid is only significant near $T_m$ at pH 1.9.
Fig. 2. Influence of the dynamics of DSPC near the gel–fluid phase transition on the aggregation kinetics of insulin. (a) DSC thermograms of pure DSPC at pH 1.9 and 7.4 with 0.1 M NaCl in the temperature range of the gel–fluid phase transition. (b) Temperature dependence of the aggregation kinetics of 0.05% (w/w) insulin in 0.1 M NaCl at pH 1.9 (upper panel) and pH 7.4 (lower panel) in the range of the respective gel–fluid transition of DSPC. Displayed are the times for half-maximum aggregation of the protein, $t_{0.5}$, at a 10-fold molar lipid excess. Samples without lipid, but under otherwise identical conditions, were used as controls.

3.2. Dependence of the aggregation kinetics on the chain length of saturated phospholipids—probing hydrophobic matching

By adding vesicles prepared from DMPC, DPPC, and DSPC, the influence of the lipid acyl chain length on the aggregation kinetics of insulin was investigated at 60°C and pH 1.9/pH 7.4 (Fig. 3). At first hand, it becomes obvious that the aggregation kinetics is drastically retarded in the NaCl-free solutions as compared to the solutions containing 0.1 M NaCl. At both pH-values, insulin carries electrical net charges (+6 at pH 1.9 and −2 at pH 7.4 (Hong et al., 2006)). The lack of charge screening in the absence of NaCl likely disfavours the approach between charged, partly unfolded insulin molecules – a prerequisite for the aggregation process – which manifests in a significant decrease of the rate of aggregation.

The result of the addition of lipid vesicles with respect to the aggregation kinetics of the protein is strongly dependent on both the pH-value and the presence of NaCl. Firstly, the additive of lipid does not significantly affect the rate of aggregation at pH 1.9 in the presence of 0.1 M NaCl, which is also observed for the other lipid systems investigated in the current study (DOPC/DOPE and DOPC/DOPS). Secondly, the addition of lipid essentially leads to a significant, three-fold acceleration of the aggregation process at pH 1.9 without NaCl, irrespective of the lipid chain length. Finally, at pH 7.4 with 0.1 M NaCl, a progressively inhibited aggregation is observed with increasing lipid chain length. The shortest-chain lipid, DMPC, does not exert any measurable effect compared to the control sample, however. Again, the bulk aggregation is rather fast due to charge screening.

3.3. Importance of the fraction of DOPE in DOPC/DOPE-vesicles for the aggregation kinetics—probing curvature elastic stress

Fig. 4 displays the dependence of the half-time for aggregation of insulin at 60°C and pH 1.9 and
7.4, respectively, on the fraction of DOPE in mixed DOPC/DOPE-vesicles. Within the experimental error, the addition of lipid vesicles at pH 1.9 only modulates the insulin aggregation kinetics when NaCl is absent. The addition of pure DOPC-vesicles to the protein solution causes a ca. two-fold acceleration of the aggregation process. In case the vesicles additionally contain DOPE, increasing fractions of DOPE progressively accelerate the aggregation process significantly. Finally, a four-fold accelerated rate of aggregation is observed compared to the control sample at the highest fraction of DOPE investigated (40%, mol/mol).

On the contrary, the addition of lipid at pH 7.4 with 0.1 M NaCl manifests in significantly decreased rates of aggregation, the extent of which becomes more prominent only with increased fractions of DOPE.

3.4. Importance of the fraction of DOPS in DOPC/DOPS-vesicles for the aggregation kinetics—probing charge interaction

Fig. 5 displays the influence of the fraction of DOPS in mixed DOPC/DOPS-vesicles on the half-time for aggregation of insulin at 60 °C and pH 1.9 and 7.4, respectively. No significant effects of the presence of DOPC/DOPS-vesicles on the aggregation kinetics of insulin can be concluded from the data at pH 1.9 in the presence of 0.1 M NaCl. In contrast, the absence of NaCl increases markedly the rate of aggregation upon addition of lipid vesicles, as compared to the control sample. Furthermore, increasing fractions of DOPS in the mixed vesicles manifest in an additional rate accelerating effect. Notably, the overall aggregation kinetics is slower compared to the other sets of measurements at pH 1.9 without NaCl. A different effect of the lipid bilayer on the insulin aggregation kinetics is observed at pH 7.4 in the presence of 0.1 M NaCl, where the addition of pure DOPC results in a marked retardation of the aggregation process. This retarding effect is, however, offset by the addition of 10–20% (mol/mol) of DOPS. Finally, the aggregation-inhibiting character of pure DOPC-vesicles vanishes in the absence of NaCl, and the aggregation speeds up progressively with increasing fractions of DOPS above approximately 20% (mol/mol).

3.5. Effect of the presence of lipid vesicles on the morphology of mature insulin aggregates

In a spot test manner, the morphology of mature insulin aggregates that form under specific pH conditions in the bulk and in the presence of lipid membranes were characterized by AFM imaging. Fig. 6 displays AFM images of insulin after overnight incubation at 60 °C in HCl, pH 1.9 without NaCl (Figs. 6a and b) and in 10 mM phosphate buffer at pH 7.4 with 0.1 M NaCl (Figs. 6c and d), both in the absence and presence of a 10-fold molar excess of DOPC/DOPE (8:2; mol/mol) vesicles. Clearly, at pH 1.9 (also with 0.1 M NaCl; data not shown) exclusively fibrillar aggregates are observed. In contrast, at pH 7.4 amorphous aggregates with heights of a few nanometres are formed. Notably, under these selected conditions, the presence of lipid vesicles does not modify the resulting insulin aggregate morphology.

4. Discussion

It is well known that lipid membranes exhibit marked discontinuities with respect to various physical parameters, such as maximum permeability, around the gel–fluid
transition temperature $T_m$ (Lasic, 1993). It is assumed that these discontinuities can be essentially explained by the maximal occurrence of line defects at the interface between fluid and gel coexisting domains due to their highly dynamic interconversion at $T_m$. In the case of insulin aggregation in the presence of membranes, line defects could favour a (partial) insertion of the partially unfolded, aggregation-prone intermediates and hence shield non-native inter-protein interactions via exposed hydrophobic patches. Such a scenario reasonably explains the observed retarded aggregation of insulin near $T_m$ of DSPC (see Fig. 2).

On the one hand, the independence of the aggregation kinetics of insulin from the lipid chain length at 60°C and 0.1 M NaCl, pH 1.9, implies that insulin does not interact strongly with lipid bilayers under these particular conditions.

A temperature of $T=60$°C and pH 1.9 in the presence of 0.1 M NaCl establish conditions where the partial unfolding of insulin molecules and – due to the screened electrostatic repulsion – aggregation are strongly favoured. Obviously, the bulk aggregation is much faster than the aggregation at the membrane surface under these conditions, so the net rate of aggregation, as extracted from the ThT-assay, is largely dominated by the rate of aggregation in the bulk solution.

The bulk aggregation in the absence of NaCl is slower by about one order of magnitude, and consequently the contribution of surface aggregation at the membrane could manifest in a clearly observable effect on the net rate of aggregation. The fact that irrespective of the lipid chain length, an accelerated protein aggregation is observed to the same extent contradicts a significant interaction of insulin with the hydrophobic interior of the model membranes under these conditions. The accelerated aggregation probably stems from attractive interactions of insulin molecules with the head group region of the membranes and/or from an entropically driven association with the membrane surface due to the release of bound water (Sharp et al., 2002). At pH 1.9, electrostatic interactions between permanent protein charges and charged lipid headgroups should only play a minor role. Although insulin carries a high positive net charge, the membrane is mainly electrically neutral due to the low pK$_a$-value of the lipid phosphate groups (pK$_a < 1$). Merely, attractive dipole–monopole, dipole–dipole and hydrogen bonding interactions could favour a reversible adsorption of partially unfolded insulin molecules at the membrane surface (Gorbenko and Kinnunen, 2006). The accompanying local concentration increase of partially unfolded insulin molecules is likely to manifest in an accelerated aggregation, because...
the initial formation of aggregation nuclei is highly concentration-dependent.

At pH 7.4, insulin carries two negative net charges, whereas the lipid molecules are zwitterionic. A reversible protein adsorption via dipole–monopole, dipole–dipole and hydrogen bonding interactions also seems realistic in this case. However, such a scenario cannot explain the systematic decrease of the rate of aggregation with increasing lipid chain length. Possibly, a partial insertion of insulin molecules into the membrane could account for the aggregation-inhibiting character of the lipids under these conditions. It is known that the transmembrane insertion of proteins can be modulated by the extent of “hydrophobic matching” in a delicate fashion (Jensen and Mouritsen, 2004).

DMPC-bilayers exhibit the smallest hydrophobic thickness among the unsaturated lipids investigated in the current study (Cantor, 1999; Winter and Pilgrim, 1989; Eisenblätter and Winter, 2006), and the present data might point towards a minimum extent of hydrophobic matching that is required for a partial membrane insertion of insulin under these conditions. As has already been discussed for the addition of saturated lipids, the insulin aggregation kinetics is obviously dominated by the aggregation kinetics in the bulk solution at pH 1.9 in the presence of 0.1 M NaCl.

The markedly accelerated aggregation of insulin in the presence of lipid vesicles at pH 1.9 without NaCl is probably caused by an unspecific surface accumulation of the protein without a significant insertion...
into the membrane interior, however. The additional aggregation-accelerating effect of DOPE might imply an increased extent of surface accumulation of insulin as compared to pure DOPC-vesicles. It is known that the presence of DOPE in DOPC-vesicles creates curvature elastic stress due to the smaller ethanolamine head group. The binding of proteins to such stressed membranes can cause the membrane to locally curvature-relax, which in turn can thermodynamically favour protein–membrane interactions in mixed DOPC/DOPE-bilayers (Stubbs and Slater, 1996; Hui, 1997; Attard et al., 2000). Analogously, the release of curvature elastic stress has been identified as a major driving force for the interaction of the amyloidogenic protein α-synuclein with highly curved small unilamellar lipid vesicles (Nuscher et al., 2004).

The inhibitory effect of the lipid vesicles at pH 7.4 in the presence of 0.1 M NaCl is slightly enhanced by the addition of DOPE. Although a surface accumulation due to stress relief might reasonably occur under these conditions, the putative counterbalancing partial membrane insertion might explain why an inhibited, instead of an accelerated, aggregation is actually observed. Notably, a similar extent of aggregation inhibition is observed for pure DOPC- and DSPC-vesicles. Whereas DSPC is slightly above $T_m$ at 60°C, DOPC is fully in the fluid phase (Koynova and Caffrey, 1998). On the other hand, DMPC is also fully in the fluid phase, but does not seem to affect the insulin aggregation kinetics at all. These observations might indicate that, besides the membrane fluidity, the exact composition of the lipid acyl chains can also modulate the extent of a putative membrane insertion of insulin, possibly via a “hydrophobic matching” mechanism. As has already been discussed for the addition of saturated lipids, the insulin aggregation kinetics is obviously dominated by the aggregation kinetics in the bulk solution at pH 1.9 in the presence of 0.1 M NaCl.

The putative surface accumulation of insulin in the presence of pure DOPC-vesicles has been pointed out earlier as a possible cause for the aggregation-accelerating effect of lipid vesicles at pH 1.9 without NaCl. The insertion of DOPS into DOPC-membranes is not expected to alter the charge distribution across the membranes drastically, because the serine carboxyl group is mainly protonated at pH 1.9. Yet, the carboxyl group could facilitate the formation of additional hydrogen bonds with insulin molecules, and a direct interaction of carboxylic acids (acetic acid, to name it) with the insulin surface has in fact been postulated under conditions resembling those in the current study (Grudzielanek et al., 2005). The complexation of exposed polar protein patches by the serine carboxyl group via hydrogen bond formation could result in an increased extent of insulin surface accumulation.

DOPS is mainly negatively charged at pH 7.4. From an exclusively electrostatic point of view, interactions between net negatively charged insulin molecules and the DOPS-containing membrane should be progressively disfavoured with increasing negative charge density. Hence, the observation that the rate of insulin aggregation approaches the value for the bulk aggregation with increasing fractions of DOPS matches the expectation. However, this effect should be more pronounced in the absence than in the presence of 0.1 M NaCl due to the enhanced charge screening in the latter case. Actually, the aggregation-accelerating potential of increased fractions of DOPS in the absence of NaCl more likely intimates an increasing extent of surface accumulation of the protein. An accelerated insulin aggregation has been observed in the presence of negatively charged lipid systems under similar conditions, at 25°C, however (Zhao et al., 2004). The corresponding authors ascribe the aggregation-accelerating effect of negatively charged lipids to a preferred surface accumulation of insulin due to attractive interactions between the net negatively charged membrane surface and locally positively charged protein patches. A somehow contradictory argumentation has been put forward by Sharp et al. who observed a faster aggregation of insulin at the membrane surface under net electrostatic repulsion than in the bulk solution (Sharp et al., 2002). The unfavourable repulsive electrostatic interactions between insulin and the membrane surface are supposed to be outweighed only by a drastic increase of the protein’s entropy, which could stem from a preferred unfolding of the protein under these conditions. The authors claim that the resulting higher fraction of aggregation-competent, partially folded insulin molecules can explain the increase of the rate of protein aggregation in such a scenario.

5. Summary

In a simplified approach to the in vivo situation, where pathogenic fibrillar protein deposits are often found associated with cellular membranes, the aggregation kinetics of insulin in the presence of various model membranes was investigated using the ThT fluorescence assay. The lipid dynamics near the gel–fluid transition, the chain length of the phospholipids and the insertion of DOPE or DOPS into DOPC-vesicles modulate the aggregation kinetics of insulin in an indifferent, an aggregation-accelerating or an aggregation-inhibiting manner, depending delicately on the pH-value and the presence of NaCl.
The rate of insulin aggregation in bulk solution seems to dominate the overall aggregation kinetics in most cases at pH 1.9 with 0.1 M NaCl. The presence of dynamic line defects and extensive volume/area fluctuations near \( T_m \) of the lipid bilayer likely facilitates a partial membrane insertion of the protein, which in turn leads to a shielding of exposed hydrophobic protein patches and hence to an inhibited aggregation. An exclusively aggregation-accelerating effect was observed in the absence of 0.1 M NaCl for all lipid additives investigated, which is likely due to an enhanced surface accumulation of the protein. Apart from weak dipole–dipole, dipole–monopole and hydrogen bonding interactions, the release of curvature elastic stress in mixed DOPC/DOPE-membranes and preferred interactions of insulin with carboxylic groups in DOPC/DOPS-membranes seem to favour an additional surface accumulation in these cases.

At pH 7.4, a partial insertion of insulin into the lipid bilayer seems to be favoured, which could account for the aggregation-inhibiting effect of all model membranes studied except those containing DOPS. Generally, the extent of inhibition increases with an approach towards the lipid’s \( T_m \), the lipid chain length and the extent of curvature stress in mixed unsaturated lipid membranes. The accelerating effect of DOPS on the aggregation of insulin under net electrostatic repulsion could not be fully explained, but might result from an increased surface accumulation and/or faster/more extensive unfolding of the protein without a subsequent membrane insertion.

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References


